ISSN: 0974-5335
IJLST (2017), 10(4):38-44

Novel Applications of Crude Bacterial BioSurfactant

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ABSTRACT
The present study was aimed to produce economically viable biosurfactant with novel applications. A total of 23 bacterial cultures isolated from mangrove sediment and garden compost samples on nutrient agar medium were tested for the prroduction of biosurfactant by growing in Mineral salt medium with 1% mineral oil as sole carbon source for 24 - 48 h and the amount of biosurfactant was quantified by the standard Orcinol method using L-rhamnose as standard. Ten isolates selected as potential based on maximum amount of biosurfactant production were used and the crude biosurfactant produced by them were further subjected for various confirmation studies such as oil spread method, hemolysis test, drop collapse test, blue agar plate method and emulsification activity. The antimicrobial (against *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli* and *Proteus vulgaris*) and anti-biofilm activities (against pre-formed *Bacillus subtilis* biofilm) of crude biosurfactant from all the ten isolates were investigated. Biosurfactant from two novel isolates identified as *Bacillus* sp and *Klebsiella* sp showed positive for maximum confirmation tests and also exhibited promising anti-bacterial and anti-biofilm activities. Thus, the present study recommends the use of crude biosurfactant as a potential alternative to chemical surfactants making their application a cost-effective and sustainable use.

Key words: biosurfactant, emulsification, antibacterial, minimal salt medium, mangrove sediment

INTRODUCTION

Surfactants are amphiphilic surface active molecules that concentrate at interfaces and decrease surface and interfacial tension. They constitute an important class of industrial chemicals widely used in almost every sector of modern industry. Great emphasis has recently been given to the environmental impacts caused by chemical surfactants due to their toxicity and difficulty in degradation in the environment. Thus exploration of natural resources has served as a precious source giving rise to the products with high value in the world market, such as biosurfactants. Biosurfactants are amphiphilic microbial molecules with hydrophilic and hydrophobic moieties. Such characteristics allow these biomolecules to play a key role in different industries. Biosurfactant production has considerable importance to the implantation of sustainable industrial processes as they can potentially replace virtually any synthetic surfactant [1, 2 &3].

The biosurfactant industry has demonstrated remarkable growth in recent decades, although the their industrial scale production remains a challenge from the economic standpoint due to high raw material costs, high processing costs and low manufacturing output. Using the knowledge of biotechnology to optimize the commercial production of this magical molecule, would definitely lay the bricks of a new and emerging biotechnology industry which would become the ultimate alternative to harmful chemicals and friendly to the environment as well. The applications of biosurfactant however, are still currently remained at the developmental stage of industrial level because of relatively high production costs [4].

Thus, the use of crude biosurfactant extract could be a novel solution, especially if the application is of environmental context, as biosurfactants in such cases do not need to be pure which would allow the creation of an economically viable technology for biosurfactant production [5 & 3]. The present investigation addresses the reduction in the production costs by the use of crude biosurfactant extract produced by bacteria and explores its novel commercial applications that make this biosurfactant a promising choice for use in environmental application.

MATERIALS AND METHODS

Screening of biosurfactant producing bacteria
Soil samples were collected from two composting unit (South Goa) and five different mangrove sediment from Chorao Island, Goa, India during the study period Dec – Feb, 2014. Samples were

aseptically collected in small plastic sterile bags using a ladle (approximately 10g) and were brought to the laboratory and kept at 4^0C prior to their processing. One gram of each soil sample was inoculated into 100 ml nutrient broth and incubated up to 48 h at 37°C. The samples were then serially diluted up to 10^{-10} dilutions with phosphate buffered saline, 0.1 ml was spread-plated on nutrient agar plates and incubated up to 48 h at 37°C. After incubation, morphologically distinct colonies were selected for screening tests.

All the bacterial cultures obtained from the above screening were inoculated in 100 ml Mineral Salt Medium (MSM) [15 g $NaNO_3$, 1.1g KCl, 1.1g NaCl, 0.00028g $FeSO_4.7H_2O$, 3.4g KH_2PO_4, 4.4g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g yeast] with 1% mineral oil as the sole carbon source and incubated for 24 and 48 h at 28 ±2°C on a rotary shaker as described by Saravanan and Vijayakumar [6]. The 24 h and 48 h grown cultures were then centrifuged separately at 5^0C at 10,000 rpm for 15 min to obtain the cell free supernatant treated as crude biosurfactant extract was quantified by the standard Orcinol method using L-rhamnose as standard [7]. To 100 µL of each sample, 900 µL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30 min at 80ºC, the samples were cooled at room temperature and the OD at 421 nm was measured. The rhamnolipid concentrations were calculated from a standard curve prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mg mL^{-1}). The cultures showing maximum production of biosurfactant were selected for further studies.

Confirmation tests for biosurfactant production
Crude biosurfactant extract obtained from 24 h old grown cultures of the selected isolates by the above mentioned method was used to perform the following preliminary screening tests. All the screening tests were performed in triplicate.

i) *Oil spread method*
The principle of this method was based on the ability of the biosurfactant to alter the contact angle at the oil-water interface. In this method, 10µl of kerosene oil was added to the surface of 50 ml distilled water in a petri dish followed by the gentle addition of 10µl crude biosurfactant extract on the center of oil layer. Displacement of oil with the formation of clear zone indicates the presence of biosurfactant. The displaced diameter is measured after 30 sec. Measured area is expressed in BS unit, known as biosurfactant unit. One biosurfactant unit (BS unit) was defined as the amount of surfactant forming 1 cm^2 of oil displaced area [8].

ii) Drop collapse test
The method was performed as described by Saravanan and Vijayakumar [6]. Tween 80 was used as a positive control. Crude oil was used in this test. 10 µl of oil was applied to the well regions of 96-well micro plates and these were left to equilibrate for 24 h. 10µl crude biosurfactant extract was transferred to the oil-coated well regions and drop size was observed after 1 min with the aid of a magnifying glass. The result was considered positive when the drop was flat and was scored as negative with round drops indicative of the lack of biosurfactant action.

iii) Emulsification activity
Emulsification activity was carried by adding 2 ml kerosene in 2 ml crude extract and vortexing for 5 min to confirm proper mixing of both the liquids and allowed to stand for 24 h and the persistent appearance of froth was taken as positive index [9].

iv) Hemolytic activity
This test is done to determine the ability of crude biosurfactant extract to induce hemolysis on blood agar by rupturing the red blood cells. Fresh crude biosurfactant extract were streaked on blood agar plate and incubated for 72 h at 30°C. Haemolytic activity was detected as the presence of a definite clear zone around the streaked area on the blood agar plate [10].

v) Blue agar plate (bap) method
Mineral salt agar medium supplemented with glucose as carbon source (1%) and cetyltrimethylammonium bromide (CTAB: 0.2 g/L) and methylene blue (0.005 g/mL) were used for the detection of anionic biosurfactant. Thirty microlitre of crude biosurfactant extract was loaded into the each well prepared in methylene blue agar plate using cork borer (4 mm). The plate was then incubated at 37°C for 48-72 h. A dark blue halo zone around the culture was considered positive for anionic biosurfactant production [9].

ISSN: 0974-5335
IJLST (2017), 10(4):38-44

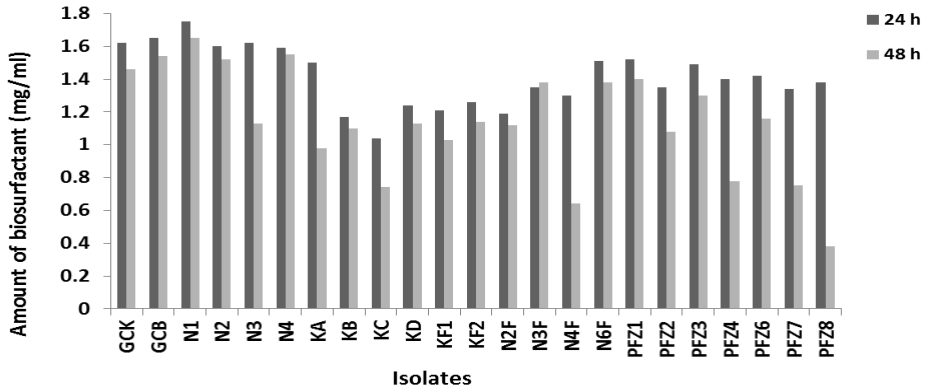

FIGURE 1 TIME COURSE OF BIOSURFACTANT PRODUCTION

TABLE 1 RESULTS OF SCREENING TESTS FOR BIOSURFACTANT PRODUCTION

Isolates	Biosurfactant (mg/ml)	Oil displacement (mm)	Drop collapse test	CTAB agar plate method	Haemolysis test	Emulsification test	Antibacterial activity				Biofilm dispersal (mean ± S.D); n=3
							E. coli	*P. vulgaris*	*B. subtilis*	*S. aureus*	
GCK	1.6	8	**+**	+	**+**	**+**	**-**	**+**	**+**	**+**	76.6 ± 0.007
GCB	1.6	10	+	-	-	-	-	-	**+**	-	65.4 ± 0.00
N1	1.7	5	-	-	-	-	+	-	**+**	-	41.6 ± 0.01
N2	1.6	8	+	-	-	-	-	-	+	-	27.0 ± 0.02
N4	1.5	5	-	-	-	-	-	-	+	+	67.0 ± 0.003
N3F	1.3	8	-	-	+/-	-	-	-	+	-	32.4 ± 0.015
N6F	1.5	6	+	+	+/-	+	-	-	+	-	74.5 ± 0.009
PFZ1	1.5	10	**+**	**+**	+	**+**	**-**	**-**	**+**	**-**	36.4 ± 0.021
PFZ3	1.4	5	-	+	-	+	-	-	+	-	70.5 ± 0.004
PFZ6	1.4	8	+	+	+	+	-	+	+	+	94.4 ± 0.004

Legend: + Positive - negative +/- doubtful

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ISSN: 0974-5335
IJLST (2017), 10(4):38-44

TABLE 2 CARBOHYDRATE UTILIZATION PROFILES OF THE BACTERIAL STRAINS GCK AND PFZ6

Test	GCK	PFZ6
Lactose	+	+
Xylose	+	-
Maltose	+	-
Fructose	+	-
Dextrose	+	-
Galactose	+	-
Raffinose	+	-
Trehalose	+	+
Melibiose	-	-
Sucrose	+	+
l- arabinose	+	+
Mannose	+	-
Inulin	+	-
Sodium gluconate	+	-
Glycerol	+	-
Salicin	+	-
Dulcitol	+	-
Inositol	+	-
Sorbitol	-	-
Mannitol	+	+
Adonitol	+	-
Arabitol	+	-
Erthritol	+	-
α – methey – D – glucoside	-	-
Rhamnose	+	-
Cellobiose	+	-
Melezitose	+	-
α – methey – D –mannoside	+	-
Xylitol	+	-
ONPG	-	-
Esculin hydrolysis	-	-
D-arabinose	-	-
Citrate utilization	-	-
Malonate utilization	-	-
sorbose	+	-

Legend: + Positive - negative

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TABLE 3 BIOCHEMICAL TESTS OF THE GCK AND PFZ6 BACTERIAL STRAINS

Tests	GCK	PFZ6
Gram characters	+	-
Endospore staining	+	-
Catalase	-	-
Citrate utilization	-	-
oxidase	+	+
Voges Proskaurer's		
Nitrate reductase	+	+
Motility test	+	_

Legend: + Positive - negative

APPLICATIONS OF BIOSURFACTANT

i) Anti-bacterial activity

The agar well diffusion assay was used to evaluate the antimicrobial activity of the crude biosurfactants against *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli* and *Proteus vulgaris* using Muller Hinton agar. About 50 µl of the respective crude biosurfactant was added to each well and incubated at 37ºC for 24 – 48 h and the zone of inhibition were recorded as a measure of anti bacterial activity of the biosurfactant [11].

ii) Biofilm degradation

Biofilm inhibition assay was carried out according to Sambanthamoorthy *et al* [12] using pre-formed *Bacillus subtilis* biofilm. To determine if the biosurfactant could disperse preformed biofilm, *B.subtilis* biofilms were established previously on a 96-well plate with pegs built into the lid that allow for the adherence and growth of biofilm. Established biofilms were exposed to crude biosurfactant extract (100 µl per well) for 1 h. Unattached organisms were removed by washing the wells three times with phosphate buffered saline. Adherent biofilms on the pegs were fixed with 200 µl of 100% ethanol prior to staining for 2 min with 100 µl of 0.3% (wt/v) crystal violet in 12% ethanol. Excess stain was rinsed off by placing the plate under running tap water. Subsequently the plates were air dried, the dye bound to the adherent microorganisms was resolubilized with 100 µl of 70% ethanol and 10% isopropanol alcohol per well. The absorbance of the dissolved dye, corresponding to the number of adherent microorganisms, was measured in a microplate reader at 595nm. The percentage reduction in adherence was calculated using the following equation:

% Reduction in adherence = [(A control) - (A sample)] /A control×100]

Experiments were performed in triplicate and the result is represented as mean ± S.D. From the above screening tests for biosurfactant, two novel isolates exhibiting maximum properties were selected and identified by morphological and biochemical tests.

RESULTS AND DISCUSSION

Twenty three bacterial strains of different colony morphology isolated from mangrove sediment and garden compost soil sample on nutrient agar plate were screened for their biosurfactant production on Mineral Salt Medium (MSM) with 1 % mineral oil (Fig 1). Ten isolates designated as GCK, GCB, N1, N2, N4, N3F, N6F and PFZ1 selected as potential based on amount of biosurfactant production were used for further studies. The time of harvest of biosurfactant was found to be after 24 h thus proving the efficacy of the medium used in this study which is cheap and economically affordable for large scale production. Mineral salt medium makes a great selective media when supplemented with hydrocarbons as a sole carbon source for growing and isolating bacteria capable of producing biosurfactants. It has been reported that hydrophobic substrates maximize the production of biosurfactant compared to hydrophilic substrates [13] which is in agreement with the present study where 1 % mineral oil was used as sole carbon source.

Confirmation of biosurfactant production through various screening methods becomes essential to select potent biosurfactant producers as proven in this present investigation. Results of various screening tests for biosurfactant production are shown in Table 1. The oil spreading assay is shown

positive by the crude biosurfactant extract from all the ten islolates to a varying degree with maximum by extract from GCK and PFZ1 isolates and is in tune with those observations reported by Anandaraj and Thivakaran [14]. The oil spreading assay is rapid and is highly sensitive to surface active compounds. Emulsification activity is one of the criteria to support the potential biosurfactant producers. Patil and Chopade [15] introduced emulsification assay as an important tool for screening the biosurfactant producers. Ellaiah *et al* [16] screened 68 isolated from soil and found only 6% with good emulsification activity where as in the present study, the crude biosurfactant extract from 50% of the isolates (isolates GCK, N6F, PFZ1, PFZ3 and PFZ6) were found to be positive for this test. The crude biosurfactant extract from the cultures GCK, GCB, N2, N6F, PFZ1 and PFZ6 showed positive for the drop collapse test. The present result is in accordance with Saravanan and Vijayakumar [6] who reported this method as sensitive and easy. The crude biosurfactant from two strains, GCK and PFZ1 showed positive for hemolysis test.

The present result is in accordance with the studies of Rashedi *et al* [17] and Anandaraj and Thivakaran [14]. But according to Youssef *et al* [18] the method has some limitations. First, the method is not specific, as lytic enzymes can also lead to clearing zones. Second, hydrophobic substrates cannot be included as sole carbon source in this assay. Third, diffusion restriction of the surfactant can inhibit the formation of clearing zones. It can give a lot of false negative and false positive results. Therefore it is recommend the blood agar method as a preliminary screening method which should be supported by other techniques. The BAP technique is highly specific and the dark blue halo zone in the methylene blue agar plate supplemented with CTAB confirmed the anionic biosurfactant production by four isolates, GCK, N6F, PFZ1 and PFZ3. The assay was developed based on the property that the concentration of anionic surfactants in aqueous solutions can be determined by the formation of insoluble ion pairs with various cationic substances. The formation of insoluble ion pair precipitates in the agar plate containing methylene blue exhibited dark blue color against the light blue background. The sensitivity of this method as one of the potent technique of screening for biosurfactant production is also reported by Satpute *et al* [9].

In the past, several studies have documented the antibacterial activity of purified biosurfactant isolated from different bacteria [12 &11]. But in the present study, the crude biosurfactant extract itself were found to be effective against the test pathogens thus enabling the economically affordable technology of application of biosurfactant. The bacterial cell membrane appears to be the target of biosurfactant activity and subsequent microbicidal activity of biosurfactant may be due to the leakage of cellular contents [19 & 20].

Biofilm infections are extremely challenging to treat because antimicrobials are less effective. The presence of biofilms causes numerous problems in the field of medicine as well as marine bioufouling; it interferes with the clinical therapy of chronic and wound-related infections as well as persistent infections of various medical devices. Although numerous strategies have been established and are currently in use to control biofilms, the search for novel, natural and effective antibiofilm agents still continues [12].

In the present study, crude biosurfactant extract from all the tested cultures showed the biofilm degradation ability to different extents (27% - 94%) (Table1). The anti-biofilm ability of the biosurfactant may be due to the two important properties that it displays, namely, antimicrobial and surfactant activity. The anti-biofilm and anti-bacterial activities of the biosurfactant observed in our studies explores the possibilities of the application of biosurfactant as alternatives to control biofilms and to prevent microbial colonization on medical devices. Crude biosurfactant exhibiting both anti bacterial and biofilm degradation capacity is however very rare [12 & 11], thus the current investigation exposes a simple method for the production of biosurfactant with novel applications.

Two novel isolates (GCK & PFZ6) producing biosurfactant exhibiting maximum properties and anti-biofilm & anti-bacterial activities were identified by morphological and biochemical tests (Table 2 & 3) as *Bacillus* sp and *Klebsiella* sp respectively according to Bergey's manual of determinative bacteriology[21].

CONCLUSIONS

The simplicity of the various techniques used in this study in screening of biosurfactant producing bacterial strains from the natural sources can contribute considerably to the reduction in the production cost of biosurfactants. The antibacterial

activity and anti-biofilm activities of the biosurfactant from *Bacillus* sp and *Klebsiella* sp. observed in these studies were promising and opens up the possibility of using them to coat surfaces of medical devices to reduce microbial growth and colonization. The production of low-cost biosurfactants is unlikely due to the complicated recovery process. But the present study explores an inexpensive and efficient technology for the production and applications of crude bacterial biosurfactant extract effective as an anti microbial and anti bio film agent.

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